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COMPLETE SPECIFICATION

Process for Purifying *B. pertussis* Antigen-containing Material

We, MERCK & CO., INC., a corporation duly organized and existing under the laws of the State of New Jersey, United States of America, of Rahway, New Jersey, United States of America, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to the separation and purification of *B. pertussis* protective antigen-containing material and the use of this material in the preparation of monovalent or polyvalent vaccines.

Whooping cough is defined as an acute, highly communicable, infectious disease caused by *B. pertussis* which in children under 4 years of age is dangerous and probably ranks first as the cause of infant mortality. Immunization against this disorder has been accomplished in the past by injection of killed cell vaccine or extracted antigen. However, the frequent incidence of side reactions such as fever, irritability, inflammation and necrosis and the rare but disheartening report of encephalitis has constituted a potent stimulus for research leading to a better vaccine.

In accordance with the present invention we have discovered that a useful vaccine for immunization against whooping cough and substantially free of undesirable side effects can be prepared by a procedure comprising mechanical disruption of *B. pertussis* cells, and separation of the bacterial cell wall material from the proto-plasmic material. This process is based on the finding that the protective antigen is an integral part of the cell wall (sometimes referred to as cell membrane or cell envelope). The protoplasm of the cell, on the other hand, possesses little or no protective activity but contains the major bulk of nitrogenous material and comprises a high concentration of toxic protein which can cause death in laboratory animals and undesirable side effects when present in vaccines that are administered clinically.

The present invention, therefore, resides in the process which comprises disrupting the intact *B. pertussis* cells, isolating the cell walls by centrifugation or by other mechanical means, and treating the isolated cell walls with a detoxifying agent which is a water miscible, C_1—C_5 monohydric alcohol, aldehyde or ketone. Preferred agents include C_2—C_4 alcohols such as ethyl, propyl or butyl alcohol, methyl ethyl ketone, acetaldehyde and, most preferably, acetone. In the preferred procedure one to five volumes of the selected ketone, aldehyde or alcohol is added to an aqueous suspension of the isolated cell wall and the mixture maintained at room temperature or below, advantageously at about −20°C., for a period up to about 24 hours. The period the mixture is held is dependent upon the temperature; at higher temperatures a shorter period suffices to make the cell wall material safe for use as a vaccine while at lower temperatures more time is needed. For example, at −20°C maximum results (i.e. safety and yields) are obtained in 18—24 hours, while at near room temperature less than an hour suffices to render the cell wall material substantially safe for use although yields of active protective antigen are sacrificed.

The *B. pertussis* cells used in the process of this invention can be grown in any one of the known, suitable media, such as charcoal agar medium, liquid media or in Cohen-Wheeler culture medium. After the growth period, the cells are harvested by centrifugation and can be used as harvested in the process of this invention or the cell paste can be frozen and stored at −30°C. until

[*Price 4s. 6d.*]

needed. If desired, the harvested cells can be lyophilized and then stored until needed, or the cells may be killed with thimerosal or by other methods that are known not to destroy the protective antigen of *B. pertussis*.

In the preferred technique of mechanical disruption the cells are resuspended in cold (2—5° C.) distilled water and subjected to explosive decompression in a Ribi Cell Fractionator (Ivan Sorvall, Inc., Norwalk, Conn.) at 30,000 p.s.i. and a temperature of 20° C. or less. While good results are obtained by employing 30,000 p.s.i. pressure, it has been found that satisfactory results are obtained when pressures ranging between about 15,000—50,000 p.s.i. are used. Other means of disrupting cells and isolating the cell walls also can be used such as by sonic oscillation or mechanical milling, but experience indicates that the Ribi Pressure Cell Fractionator produces the highest yields of clean wall material. Before use, the Ribi Cell Fractionator is sterilized advantageously by exposing all of its tubing to ethylene oxide over a 24-hour period and then flushing with sterile nitrogen gas. The pressure unit is sterilized for one hour preferably by bringing all of its parts into contact with β-propiolactone (0.5%) and then flushing with sterile distilled water. A multiphase bacterial retaining filter or other suitable filter connects the decompression chamber with a nitrogen tank.

The *B. pertussis* cell suspension is fed into the compression chamber of the Fractionator where it is subjected to a pressure of 15,000—50,000 p.s.i. which forces the cells into the decompression chamber where they explosively rupture. The decompression chamber is maintained at 20° C. or less by precooled nitrogen gas which is passed through the multiphase filter and into the chamber. The effluent from the decompression chamber containing the cell walls and protoplasm is collected in a sterile container immersed in an ice bath.

The effluent containing the disrupted cell material is centrifuged, the supernatant fluid discarded and the cell wall material remaining can be washed with cold distilled water to eliminate highly toxic, soluble protoplasmic constituents. Either washed or unwashed cell walls are suspended in distilled water to give a concentration equivalent to from 100 B/ml. to 2,000 B/ml. (Bacteria/ml), although for practical purposes a concentration equivalent to 500 B/ml. generally is used. The wall concentrate then is mixed slowly with the detoxifying agent and stored preferably at −20° C. for 18—24 hours. Although higher temperatures have been used successfully, the lower temperatures result in higher yields of active protective antigen. The treated walls are next sedimented by centrifugation and may be washed with cold distilled water. The sediment is resuspended with cold distilled water to an equivalence of 500 B/ml. and homogenized. Homogenization can be accomplished by passage through a sonic oscillator such as the Raytheon (Registered Trade Mark) 10 KC or by means of various blenders or mills.

The suspension of treated cell walls is next preserved with an effective amount of preservative, such as thimerosal, benzethonium chloride, benzyl alcohol, gamma picolinium chloride or other known acceptable preservative and diluted to any desired strength, preferably to a strength of at least 8 protective units of *B. pertussis* antigen per ml.

The detoxified cell walls containing protective antigen obtained by the novel method of this invention can be used to prepare an aqueous vaccine or the wall material containing antigen may initially be adsorbed on to adjuvants such as aluminium hydroxide or phosphate or precipitated with alum and then made up as a vaccine, for example by suspending the adsorbate or alum precipitate in physiological saline at a concentration of at least 8 protective units per ml. As the protective antigen is compatible with other antigens and/or toxoids, it can be combined with them in the conventional manner to provide a polyvalent vaccine in unitary dosage form.

The present invention has a number of distinct advantages. The product is free of all protoplasmic constituents one of which, the heat-labile toxin, is known to be extremely toxic for laboratory animals and may be responsible for some of the side reactions in humans. The product is free of nutrient medium and extraneous chemical substances. The product is compatible with other antigenic materials currently combined with intact pertussis cell antigen. The process is simple and easily carried out and is reproducible giving high yields of active material.

The process is described in detail in the following example. It is to be understood that modifications can be made in the various techniques employed in carrying out the process of this invention; the important contribution being the separation and detoxification with the aforesaid agents of cell walls containing the protective antigen of *B. pertussis* that have been isolated from protoplasm as well as from other extraneous substances.

EXAMPLE 1

The cells from a 48-hour culture of *B. pertussis*, grown on Cohen-Wheeler medium, are harvested by adjusting the medium to pH 7.0 with concentrated hydrochloric acid, and centrifuging in a Sharples (Registered Trade Mark) centrifuge. The cell paste obtained is resuspended in distilled water, passed through a 200-mesh nylon screen to remove gross particles and the filtrate is adjusted to a concentration equivalent to 500 B/ml. The cell concentration preferably

should be equivalent to 100—1000 B/ml. for the purposes of this invention.

The cell concentrate (1600 ml.) is fed into a pre-sterilized Ribi Cell Fractionator and subjected to a pressure of 30,000 p.s.i. The cells under pressure are extruded into the decompression chamber, maintained at 20° C. or less by cooled nitrogen gas, where the cells rupture explosively. The cellular material drains from the decompression chamber into a sterile vessel immersed in an ice bath, yielding 1600 ml. of effluent.

The effluent is centrifuged in a Spinco 18,000 batch bowl rotor head at 16,000 r.p.m. for 3 hours ("Spinco" is a Registered Trade Mark). Other centrifuging equipment can be used, such as the Sharples Centrifuge, for example. The supernate is decanted from the rotor head and the head may be refilled and spun one or more times, if desired, without removing the residue. The supernate that is removed and discarded contains the highly toxic protoplasmic fraction of the cell concentrate.

The residue or sediment remaining in the rotor head is removed preferably by adding steel beads to the rotor head and gently agitating the head by placing it on a rolling mechanism. Other known methods also can be used to dislodge the residue from the rotor head.

The residue is washed out of the rotor head with cold distilled water (2—5° C.) (approx. 1600 ml.); 3 volumes of acetone (maintained cold by a dry ice and acetone bath) is added with constant stirring and the mixture is stored at −20° C. for 18 hours. The concentration of cell wall and acetone can as well be adjusted to a pertussis concentration equivalent to 100—2,000 B/ml. The material is next centrifuged at 2000 r.p.m. for 30 minutes and the supernatant discarded. The sediment is washed twice with cold distilled water (a volume equal to the discarded supernatant) by centrifugation and resuspended in cold sterile distilled water to an equivalence of 500 B/ml. The washed sediment is then homogenized by passage through a Raytheon sonic oscillator (10 KC) for 5 minutes. The washed sediment is resuspended in sterile buffered saline pM 7.2 to a volume of 1600 ml. It can be stored indefinitely as such as 2—5° C. This preparation can be diluted according to the standards prescribed by the U.S. National Institute of Health (N.I.H.) i.e. to contain at least 8 protective units per ml, and used to prepare aqueous adjuvant or multivalent vaccines.

EXAMPLE 2

Adjuvant vaccine

Sterile 10% by weight aqueous potassium alum solution (178 ml.) is added slowly while shaking the suspension of cell wall material from Example 1, and the mixture is stored at 2—5° C. for 18 hours. The clear fluid above the flocculated sediment may be decanted and/or the sediment may be centrifuged at 1000 to 2000 r.p.m. in the cold and the supernatant liquid discarded. The sediment is washed twice by centrifugation with volumes of cold distilled water equal to 2 to 5 times the volume of the sediment and the washed sediment is resuspended in sterile buffered saline pH 7.2 to a volume of 3100 ml. at an equivalence of 258 B/ml. The vaccine concentrate is adjusted to 32 B/ml. equivalent or less by addition of sterile saline, pH 7.2, or 0.3M glycine buffer. Thimerosal is added as a preservative in an amount sufficient to produce a concentration of 1:10,000 when diluted. This vaccine remains stable when stored at 2—5° C.

The alum flocculated wall preparation when diluted to an equivalence of 32 B/ml., contains 15.7 protective units (P.U.) per total human dose (1.5 ml.) compared with the N.I.H. No. 6 control having 12 protective units per total human dose and can be used to prepare adjuvant or multivalent vaccines.

The potency of the wall preparation and the N.I.H. control is determined by administering serial dilutions of the extract and the control to separate groups of mice and then challenging the animals intracranially with 0.03 ml. of a dilution of known virulent *B. pertussis* containing 10^5 organisms/ml. The results of replicate potency assays are given below:

Assay*	P.U./T.H.D.**
1	9.0
2	11.0
3	12.75
4	14.4
5	20.25
6	27.0
Mean:	15.7

* Vaccine equivalent 32 B/ml.
** Total Human Dose = 1.5 ml.

The adjuvant vaccine of Example 2 was found to be non-toxic according to N.I.H. standards that require that, upon intraperitoneal injection of 1/2 of a single human dose into mice, there be no loss of weight in 3 days, a net gain of 3 grams in 7 days, and no more than 5% mortality in the animals used in the study. Upon I.P. injection of 0.25 ml. of the protective antigen extract from Example 2 into 10 mice, an average weight gain of 2.4 grams was observed at the end of 3 days, and an average gain of 4.7 grams was observed at the end of 7 days, with no deaths at the end of the test period.

The vaccine of Example 2 also passed the animal safety test in that no deaths and

no symptoms occurred when 1/2 of a single human dose was injected into each of two 20 gram mice and 3 times a single human dose was injected into each of two 350 gram guinea pigs.

Conventional sterility tests established that the vaccine was free of mould and bacteria.

Electrophoretic and Ouchterlony analysis established the vaccine of Example 2 was devoid of the major portion of antigens other than protective antigen found in the intact whole cell. The final vaccine contains one-third or less of the total nitrogen found in conventional vaccines of this type.

EXAMPLE 3
Aqueous Vaccine

The protective antigen concentrate from Example 1 is adjusted with buffered saline (pH 7.2) containing thimerosal 1:10,000 to a final concentration of 32 B/ml. or less. This vaccine remains stable when stored at 2—5° C.

EXAMPLE 4
Multivalent Vaccines

Toxoids, such as diphtheria and tetanus toxoids, can be added to either of the above aqueous or adjuvant vaccines to give a multivalent vaccine wherein the toxoids are present in concentrations normally recommended.

The invention contemplates the separation of protective antigen from *B. pertussis* in the form of cell walls by employing modifications of the procedures illustrated in Example 1 that have been described above. The concentrate of protective antigen thus obtained can be adjusted to any desired concentration and made up into a monovalent or polyvalent vaccine by any of the methods conventionally employed for preparing vaccines particularly those containing *B. pertussis* antigen. Additional examples to illustrate working within the above preferred ranges are not provided as it will be evident to those skilled in the biological sciences how to make the various modifications specifically taught herein.

WHAT WE CLAIM IS:—

1. A process for the separation and purification of *B. pertussis* antigen-containing material prior to use as a vaccine, which comprises mechanically disrupting *B. pertussis* cells, separating the cell wall material from the protoplasmic material and treating the isolated cell wall material in aqueous suspension at room temperature or below with a detoxifying agent, which is a water miscible, C_1—C_3 monohydric alcohol, aldehyde or ketone.

2. A process according to claim 1, wherein the cell wall suspension is treated with the detoxifying agent at about —20°C for from 18—24 hours.

3. A process according to claim 1 or 2, wherein the detoxifying agent is used in an amount of from 1—5 volumes per volume of the cell wall suspension.

4. A process according to claim 1, 2 or 3, wherein the detoxifying agent used is acetone.

5. A process according to any one of the preceding claims, wherein the suspension of cell wall material used has a *B. pertussis* concentration equivalent to 100 to 2,000 B/ml.

6. A process according to claim 5, wherein the suspension of cell wall material used has a *B. pertussis* concentration equivalent to about 500 B/ml.

7. A process according to any one of claims 1—6, wherein the *B. pertussis* cells are ruptured by feeding a suspension the cells at a pressure of from 15,000 to 50,000 p.s.i. into a cooled decompression chamber.

8. A process according to claim 7, wherein the cell wall material is separated from protoplasmic material by centrifuging.

9. A process according to any one of the preceding claims including the further step of sedimenting the treated cell wall material and resuspending the treated cell wall material in distilled water.

10. *B. pertussis* antigen-containing material when prepared by a process claimed in any one of the preceding claims.

11. Vaccine containing *B. pertussis* antigen-containing material according to claim 10.

12. Vaccines according to claim 11, containing at least 8 protective units per ml. of *B. pertussis* protective antigen.

13. A process for the preparation of a vaccine which comprises adsorbing *B. pertussis* antigen-containing material prepared by a process claimed in any one of claims 1—9 with an adjuvant, which is aluminum hydroxide or aluminum phosphate, or precipitating such material with alum, and resuspending the adjuvant absorbate or alum precipitate in physiological saline at a concentration of at least 8 protective units of *B. pertussis* antigen per ml.

14. A process according to claim 13, wherein there is added to the vaccine at least one bacterial toxoid or compatible antigen.

15. A process according to claim 13 or 14, in which thimerosal is added to the vaccine to a final concentration of at least 1:10,000.

For the Applicants,
D. YOUNG & CO.,
Chartered Patent Agents,
9, Staple Inn,
London, W.C.1.

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